Oral glutamine attenuates indomethacin-induced small intestinal damage

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A B S T R A C T

The use of NSAIDs (non-steroidal anti-inflammatory drugs), although of great therapeutic value clinically, is limited by their tendency to cause mucosal damage in the gastrointestinal tract. In the small intestine, the effects these drugs have been shown to produce include inhibition of cyclo-oxygenase, mitochondrial dysfunction and free radical-induced oxidative changes, all of which contribute to the mucosal damage seen. Glutamine is a fuel preferentially used by enterocytes and is known to contribute to maintaining the integrity of these cells. In the present study, we investigated the effect of glutamine on indomethacin-induced changes in the small intestinal mucosa. Rats were given 2% glutamine or glutamic acid or isonitrogenous amino acids, glycine or alanine, in the diet for 7 days. Indomethacin was then administered orally at a dose of 40 mg/kg of body weight. After 1 h, the small intestine was removed and used for the measurement of parameters of oxidative stress and mitochondrial and BBM (brush border membrane) function. Evidence of oxidative stress was found in the mucosa of the small intestine of drug-treated rats, as indicated by significantly increased activity of xanthine oxidase ($P < 0.001$) and myeloperoxidase ($P < 0.001$), with corresponding decreases in the levels of several free radical scavenging enzymes and α-tocopherol ($P < 0.001$ in all cases). Levels of products of peroxidation were also significantly elevated ($P < 0.001$ for all the parameters measured). In addition, oxidative stress was evident in isolated intestinal mitochondria and BBMs ($P < 0.001$ for all the parameters measured), with associated alterations in function of these organelles ($P < 0.001$ for all the parameters measured). Supplementation of the diet with glutamine or glutamic acid prior to treatment with indomethacin produced significant amelioration in all the effects produced by the drug in the small intestine ($P < 0.001$ for all the parameters measured). Glycine and alanine were found to be much less effective in these respects.

INTRODUCTION

NSAIDs (non-steroidal anti-inflammatory drugs) are used extensively in clinical medicine as analgesics, antipyretics and anti-inflammatory agents. They are known to exert their therapeutic effects through inhibition of COX (cyclo-oxygenase) [1], a key enzyme in the formation of prostaglandins. In spite of their therapeutic utility, however, these drugs show a tendency to cause mucosal damage in the gastrointestinal tract [2], a circumstance that limits their use. The resultant decrease in cellular levels of prostaglandins, associated with the use of these drugs, is also widely held to be the basis of the gastric toxicity caused by them. This does not, however, fully explain the mechanism of damage in the small intestine [3]. One of the hypotheses advanced to

Key words: glutamine, indomethacin, non-steroidal anti-inflammatory drug (NSAID), oxidative stress, small intestine.
Abbreviations: BBM, brush border membrane; KH, Krebs–Henseleit; MDA, malondialdehyde; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; XDH, xanthine dehydrogenase; XO, xanthine oxidase.
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explain the pathogenesis of NSAID-induced enteropathy involves the action of the drug in uncoupling or inhibiting oxidative phosphorylation. Indomethacin, a commonly used NSAID, has been shown to have these effects in isolated liver mitochondrial preparations [4]. This finding has been corroborated by studies that have shown decreases in the energy charge in jejunal tissue *in vitro* in the presence of the drug [5].

Other factors have also been shown to be involved in the pathogenesis of NSAID-induced damage in the intestine. Oxidative stress is one such putative mechanism [6]. Earlier work on the effects of indomethacin on the small intestine has shown that the drug produces free radical-induced damage and mitochondrial dysfunction in the enterocytes, with the villus tip cells being particularly susceptible to such effects [7]. Oxidative stress has also been reported in the intestinal BBMs (brush border membranes), leading to structural and functional impairment in these membranes [8].

Glutamine is one of the most abundant free amino acids in the body. It acts as a major metabolic fuel for rapidly dividing cells, such as intestinal epithelial cells and cells of the immune system. It plays a role in protecting against mucosal atrophy seen in prolonged states of parenteral nutrition, in the healing of gastrointestinal mucosa after damage due to either radio- or chemotherapy, improving gut and systemic immune function and reducing episodes of bacterial translocation [9,10]. It is also known to play a role in signal transduction and cellular responses involving stress and anti-apoptotic genes [11]. In addition, glutamine has been shown to attenuate the leucocyte adhesion and increase in intestinal permeability caused by NSAIDs [12,13]. In the present study, we investigated the effects of oral glutamine supplementation on indomethacin-induced changes in the small intestine.

MATERIALS AND METHODS

Materials

Indomethacin, arsenazo-III, Hepes, Trypan Blue, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], DMSO, ADP, succinate, Tris/HCl, CDNB (1-chloro-2,4-dinitrobenzene), DPNH (2,4-dinitrophenylhydrazine), *O*-dianisidine dihydrochloride, EDTA, BSA, DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid], NAD, NADPH, GSSG (oxidized glutathione), GSH (reduced glutathione), TBA (2-thiobarbituric acid) and xanthine were obtained from Sigma. PEG (polyethylene glycol) 4000 was obtained from Fluka. [^{14}C]Glucose was obtained from Bhabha Atomic Research Center (Bombay, India). All other chemicals used were of analytical grade. Millipore membranes (pore size, 0.45 μm) were obtained from Millipore, India.

Animals

Male albino rats (200–250 g), exposed to 12 h light/dark cycles and fed with water and rat chow *ad libitum*, were used for the experiments. All the procedures performed on the animals had been approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Protocol for administration of experimental compounds

Male rats were fasted overnight and treated with indomethacin (40 mg/kg of body weight) by gavage [7,8]. Control animals received an equal volume of the vehicle for the drug (5 % sodium bicarbonate). After 1 h, the animals were killed by cervical dislocation, their abdomens were opened immediately and the entire length of the small intestine was removed.

For studies on the effect of pretreatment with various amino acids, the following protocols were followed. Rats were placed on powdered rat chow supplemented with 2 % (w/w) glutamine for 7 days. The same concentrations of glutamic acid and isonitrogenous amino acids, glycine or alanine, were also administered for the same duration for the purpose of comparison. On day 8, the rats were treated with indomethacin and, 1 h later, the animals were killed. Each experimental group used consisted of six individual animals.

Isolation of enterocytes

Total enterocytes were isolated from the small intestine by the metal chelation method [14]. Briefly, the intestine was washed with KH (Krebs–Henseleit) buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4 and 1.9 mM $MgSO_4$, pH 7.4), filled with the same buffer containing 5 mM EDTA and 0.25 % (w/v) albumin and incubated at 37 °C for 12 min in a beaker containing KH buffer alone. After incubation, the intestine was washed with ice-cold KH buffer containing 0.25 % (w/v) albumin and then filled with the same buffer. Enterocytes were detached by gently rubbing the intestine along its entire length. Following this, the intestinal fluid containing detached cells was centrifuged at 900 *g* for 5 min. The cells in the pellet obtained were washed twice and the final preparation of enterocytes was suspended in 25 mM PBS (pH 7.4).

Assessment of cellular respiration

Cell viability of the isolated enterocytes was assessed by dye-exclusion method using Trypan Blue [15]. An aliquot of the cell suspension was mixed with 0.1 % Trypan Blue in PBS. A portion (10 μl) of this mixture was placed in a Neubar chamber. Both the total and viable number of cells were counted and the percentage of viable cells was calculated. The degree of MTT reduction by the cells was measured as described previously [16].

Isolation of mitochondria

Mitochondria were isolated from the enterocytes by differential centrifugation as described previously [17]. The purity of the preparation was checked by enrichment of the marker enzyme succinate dehydrogenase [18].

Preparation of mucosal homogenate and assay of enzymes

The small intestine was opened up along its length and the mucosa scraped using a glass slide. This material was used for the preparation of 3 % homogenates using Tris/mannitol buffer [2 mM Tris/HCl (pH 7.1) containing 50 mM mannitol] as described previously [19].

MPO (myeloperoxidase) activity was measured as described previously [20], with certain modifications. The homogenate was first treated with hexadecyltrimethyl ammonium bromide to a final concentration of 0.5 % of the compound and centrifuged at 1000 *g* for 4 min. The supernatant obtained was used for the measurement of MPO activity. The assay system consisted of 50 mM potassium phosphate buffer, pH 6, 0.167 mg of *o*-dianisidine, 0.1 μM H_2O_2 and an aliquot of the enzyme. The rate of decomposition of H_2O_2 by MPO with *o*-dianisidine as the hydrogen donor was determined by measuring the rate of colour development at 460 nm.

Activities of XO (xanthine oxidase) [21], XDH (xanthine dehydrogenase) [21], catalase [22], glutathione peroxidase [23], glutathione reductase [24] and SOD (superoxide dismutase) [25] were measured in the homogenate as described previously.

Isolation of BBM vesicles

BBM vesicles were prepared from the mucosal scrapings [19]. Purity of the preparation was checked by enrichment of the marker enzyme alkaline phosphatase [26].

Assessment of mitochondrial function

Various parameters of mitochondrial function were assessed in the isolated organelles. Oxygen uptake was determined polarographically using a Clark type electrode (YSI Model 5300 Biological Oxygen Monitor) [27]. MTT reduction was determined using a microtitre plate as described previously [16]. The amount of MTT formazan formed was calculated using the molar absorption coefficient of MTT formazan ε_{570} of 17 000 M^{-1} cm^{-1} at pH 7.4–8.0. Permeability of the inner mitochondrial membrane was determined by measuring the decrease in absorbance at 540 nm [16]. Calcium uptake by the mitochondria was followed by measuring the changes in absorption spectrum of arsenazo-III [28].

Assessment of parameters of oxidative stress

Homogenates were used for measurements of various parameters of oxidative stress. MDA (malondialdehyde) content was measured as described previously with minor modifications [29]. The mixture consisted of 0.8 ml of sample (corresponding to approx. 1 mg of protein), 0.2 ml of 8.1 % (w/v) SDS, 1.5 ml of 20 % acetic acid and 1.5 ml of 0.8 % TBA (2-thiobarbituric acid). The mixture was heated in a boiling water bath for 60 min. The absorbance of the supernatant was read at 532 nm. The amount of lipid peroxides present that reacted with TBA was calculated from a standard curve prepared using 1,1′,3,3′ tetramethoxypropane.

Other parameters measured in the homogenate were conjugated dienes [30], protein carbonyl [31], thiols [32] and α-tocopherol [33]. These parameters were also measured in mitochondrial and BBM suspensions. Protein was estimated by Lowry's method [34], using BSA as standard.

Measurement of D-glucose uptake

Isolated BBMs were assessed for their ability to transport glucose by the rapid filtration technique as described previously [35]. Briefly, 50 μl of BBM vesicles corresponding to 100 μg of protein were incubated with 150 μl of uptake buffer {150 mM sodium isothiocyanate, 50 μM D-glucose, 0.8 μCi [^{14}C]D-glucose and 10 mM Hepes (pH 7.5)} for various time periods (10, 20, 30, 60, 120 and 300 s). At the end of the incubation, the mixture was diluted with 2 ml of ice-cold stop buffer [150 mM NaCl, 10 mM Hepes and 0.2 mM phloridzin (pH 7.5)] and immediately filtered under constant vacuum. The filter was washed three times with 5 ml of stop buffer and transferred to counting vials. The radioactivity retained in the filter was counted using on LKB Rack-Beta scintillation counter.

Statistical analysis

Data were analysed using ANOVA, with Bonferroni correction for multiple Student's *t* test as a post hoc test, to look for differences in the means of the various experimental groups. A P value < 0.05 was taken to indicate significance. Data analysis was carried out using Statistical Package for the Social Scientist (SPSS), version 11.

RESULTS

Effects on enterocytes

Viability of and MTT reduction by the enterocytes were significantly decreased in indomethacin-treated cells compared with control cells (Figures 1A and 1B). Prior treatment with glutamine or glutamic acid restored these parameters to near control values. Such reversal of the effects of the drug was not seen with either glycine or alanine pretreatment.

Measurement of activity of enzymes in the mucosal homogenate showed that there was a significant increase

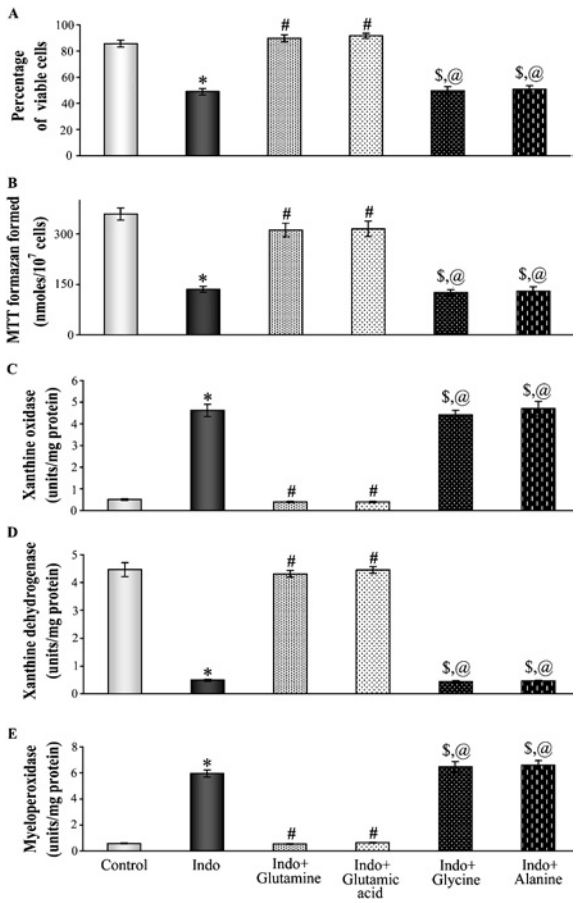

Figure 1 Viability of (A) and MTT reduction by (B) total enterocytes and XO (C), XDH (D) and MPO (E) activities in mucosal homogenates isolated from control rats and rats treated with indomethacin in the absence or presence of the different pretreatments

Each value represents means ± S.D. ($n = 6$ rats). (A–E) Overall P value for between-group comparison was < 0.001. $^{*}P < 0.001$ compared with control; $\#P < 0.001$ compared with indomethacin (indo); $\$P < 0.001$ compared individually with indo + glutamine and indo + glutamic acid. (A–D) $@P = 1$ compared with indo. (E) $@P = 0.03$ and $P = 0.003$ when indo compared with indo + glycine and indo + alanine respectively.

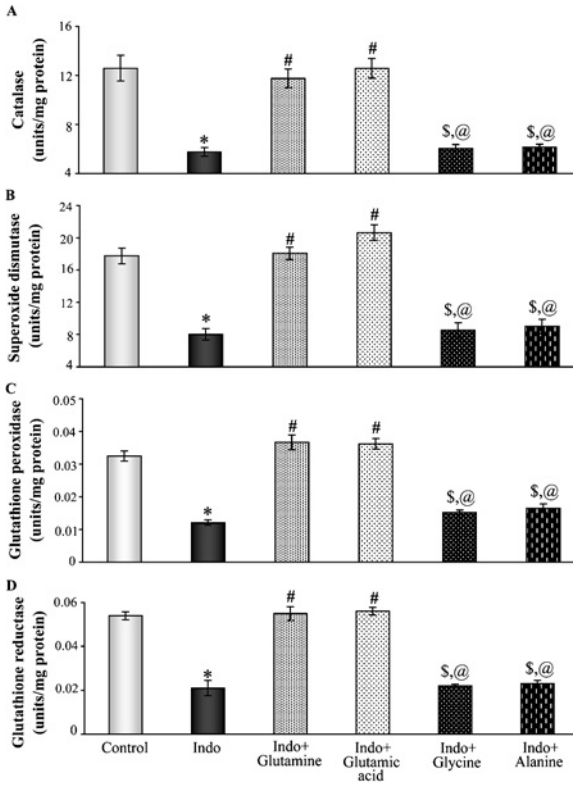

Figure 2 Activities of (A) catalase, (B) SOD, (C) glutathione peroxidase and (D) glutathione reductase in mucosal homogenates from control rats and rats treated with indomethacin in the absence or presence of the different pretreatments

Values represent means ± S.D. ($n = 6$ rats). (A, B and D) Overall P value for between-group comparison was < 0.001. (C) Overall P value for between-group comparison was < 0.01. $^{*}P < 0.001$ compared with control; $\#P < 0.001$ compared with indomethacin (indo); $\$P < 0.001$ compared individually with indo + glutamine and indo + glutamic acid. (A, B and D) $@P = 1$ compared with indo. (C) $@P = 0.017$ and $P < 0.001$ when indo compared with indo + glycine and indo + alanine respectively.

in the activity of XO (Figure 1C) accompanied by a concomitant decrease in XDH in the drug-treated rats (Figure 1D). The activity of MPO was also significantly higher in the homogenates from these animals (Figure 1E). These effects were negated by pretreatment with glutamine or glutamic acid. Administration of alanine or glycine did not abolish the changes seen in response to the drug.

Parameters of oxidative stress

Measurement of the free radical scavenging enzymes revealed that the activities of catalase (Figure 2A), SOD (Figure 2B), glutathione peroxidase (Figure 2C) and glutathione reductase (Figure 2D) were significantly lower in the homogenates from drug-treated rats compared with those from control animals. All these changes were abolished by pretreatment with glutamine or glutamic acid, but not by glycine or alanine.

Assessment of indicators of oxidative stress showed significant increases in the levels of MDA, conjugated dienes and protein carbonyl and decreases in α-tocopherol and thiols in rats treated with indomethacin when compared with control animals (Table 1). All these changes were abolished by pretreatment with glutamine or glutamic acid. Pretreatment with glycine or alanine ameliorated the effects of indomethacin in the case of some of the parameters, but these effects were much lower in magnitude than those seen with glutamine or glutamic acid (Table 1).

Table 1 Levels of malondialdehyde, conjugated dienes, protein carbonyls, α-tocopherol and thiols in homogenates, mitochondria and BBMs from control rats and rats treated with indomethacin in the absence or presence of the different pretreatments

Values are means $\pm$ S.D. ($n = 6$ rats). Overall P values for between-group comparisons were < 0.01. $^{*}P < 0.001$ compared with control; $\#P < 0.001$ compared with indomethacin (indo) alone; $\$P < 0.001$ compared with indo + glutamine; $@P < 0.001$ compared with indo + glutamic acid; $\dagger P = 1$ compared with indo; $\ddagger P > 0.001 < 0.05$ compared with indo; $\S P > 0.051 < 1$ compared with indo.

Parameter	Control	Indo	Indo + glutamine	Indo + glutamic acid	Indo + glycine	Indo + alanine
Homogenate						
MDA (nmol/mg of protein)	1.84 ± 0.12	8.05 ± 0.52*	1.80 ± 0.04#	1.86 ± 0.19#	6.51 ± 0.3#$@	7.52 ± 0.63$@§
Conjugated dienes (nmol/mg of protein)	34.12 ± 2.9	67.21 ± 3.13*	35.12 ± 1.52#	33.75 ± 1.99#	63.98 ± 3.17$@§	59.83 ± 3.29$@†
Protein carbonyl (nmol/mg of protein)	26.03 ± 2.03	59 ± 3.96*	27.91 ± 0.96#	30.58 ± 2.03#	55.57 ± 2.55$@§	57.19 ± 1.41$@†
α-Tocopherol (nmol/mg of protein)	9.42 ± 0.87	3.45 ± 0.12*	10.65 ± 0.91#	110.02 ± 10.01#	4.15 ± 0.15$@†	5.18 ± 0.35$@‡
Total thiols (nmol/mg of protein)	29.59 ± 3.03	15.07 ± 1.08*	30.74 ± 1.59#	32.09 ± 2.28#	19.34 ± 2.29$@‡	18.38 ± 2.46$@§
Mitochondria						
MDA (nmol/mg of protein)	2.25 ± 0.12	7.3 ± 0.2*	3.2 ± 0.41#	2.9 ± 0.41#	7.99 ± 0.88$@§	8.87 ± 0.78#$@
Conjugated dienes (nmol/mg of protein)	20.07 ± 1.41	65.85 ± 3.89*	21.33 ± 1.27#	23.56 ± 1.29#	56.9 ± 3.88#$@	57.29 ± 3.17#$@
Protein carbonyl (nmol/mg of protein)	20.86 ± 1.01	59.35 ± 1.32*	21.5 ± 1.93#	21.97 ± 1.34#	60.99 ± 3.37$@†	57.41 ± 1.71$@†
α-Tocopherol (nmol/mg of protein)	10.47 ± 0.9	4.75 ± 0.24*	11.35 ± 0.11#	12.35 ± 0.94#	3.45 ± 0.31$@§	4.65 ± 0.35$@†
Total thiols (nmol/mg of protein)	25.95 ± 2.17	15.46 ± 1.15*	30.08 ± 2.17#	28.88 ± 1.1#	17.2 ± 1.65$@†	19.37 ± 1.04$@‡
BBM						
MDA (nmol/mg of protein)	1.75 ± 0.106	6.54 ± 0.36*	1.79 ± 0.21#	1.77 ± 0.15#	5.65 ± 0.58$@‡	6.19 ± 0.72$@†
Conjugated dienes (nmol/mg of protein)	0.317 ± 0.01	0.58 ± 0.02*	0.30 ± 0.02#	0.299 ± 0.02#	0.517 ± 0.05$@‡	0.528 ± 0.02$@‡
Protein carbonyl (nmol/mg of protein)	22.44 ± 2.25	59.37 ± 2.25*	23.07 ± 2.78#	23.63 ± 1.64#	53.19 ± 2.95#$@	51.83 ± 1.53#$@
α-Tocopherol (nmol/mg of protein)	0.22 ± 0.02	0.13 ± 0.01*	0.23 ± 0.02#	0.235 ± 0.01#	0.166 ± 0.01$@†	0.109 ± 0.01$@†
Total thiols (nmol/mg of protein)	24.99 ± 2.06	14.02 ± 1.06*	28.27 ± 1.88#	26.98 ± 1.81#	15.91 ± 1.37$@§	17.41 ± 1.01$@‡

Effects on mitochondria

Changes in mitochondrial indices were observed after indomethacin treatment, including significant decreases in respiratory control ratio (Figure 3A), MTT reduction (Figure 3C) and calcium uptake (as evidenced by the reduced fall in optical density of the calcium–arsenazo complex; Figure 3D) and increased mitochondrial swelling (as indicated by decreased absorbance at 540 nm; Figure 3B). These effects were negated by prior administration of glutamine or glutamic acid, but not with glycine or alanine.

Oxidative stress was found to occur in these mitochondria, as shown by significantly elevated levels of MDA, conjugated dienes and protein carbonyls with significant decreases in levels of α-tocopherol and thiols. Pretreatment with glutamine or glutamic acid abolished these changes (Table 1). The administration of glycine or alanine ameliorated the effects of indomethacin in the case of some of the parameters, but these effects were much lower in magnitude than those seen with glutamine or glutamic acid (Table 1).

Effects on BBMs

In BBMs, the functional ability to transport glucose across the membranes (Figure 4) was found to be decreased after indomethacin treatment. This decrease was statistically significant at 20 s after incubating BBMs with radiolabelled glucose, but not at the other time periods measured. Pretreatment with glutamine and glutamic acid, but not glycine and alanine, restored the functional ability of BBMs to transport glucose.

Significant increases were seen in the levels of products of peroxidation with a decrease in levels of antioxidants in BBMs from drug-treated animals (Table 1). Pretreatment with glycine and alanine resulted in amelioration of some of these effects, but protection afforded by glutamine or glutamic acid was much greater and consistent for all parameters measured (Table 1).

DISCUSSION

Glutamine is the most abundant amino acid in the body. The small intestine is the principal organ of glutamine consumption, extracting approx. 20–30 % of circulating glutamine in the post-absorptive state. In fact, it uses glutamine as an oxidative fuel in preference to glucose [36]. Uptake of glutamine by the enterocytes occurs from the gut lumen across the BBM and from the blood stream via capillaries that are adjacent to the basement membrane. This avid uptake of glutamine by the mucosal cells is due, in part, to the high activity of the enzyme glutaminase, leading to the conversion of glutamine into glutamic acid with the release of ammonia [37]. Glutamic acid is transaminated and then oxidized

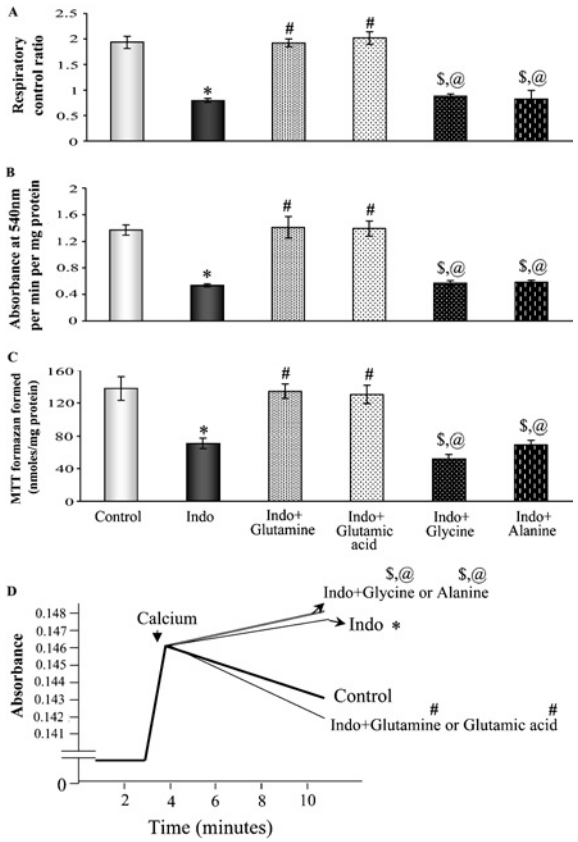

Figure 3 Respiratory control ratios (A), absorbance at 540 nm (B), MTT reduction (C) and changes in absorbance due to the concentration of calcium in the medium (D) in mitochondria isolated from total enterocytes from control rats and rats treated with indomethacin in the absence or presence of the various pretreatments

Values represent means ± S.D. ($n = 6$ rats). (A–D) Overall P value for between-group comparison was < 0.001. $^{*}P < 0.001$ compared with control; $\#P < 0.001$ compared with indomethacin (indo); $\$P < 0.001$ compared individually with indo + glutamine and indo + glutamic acid. (A, B and D) $@P = 1$ compared with indo. (C) $@P = 0.023$ and $P = 1$ when indo compared with indo + glycine and indo + alanine respectively.

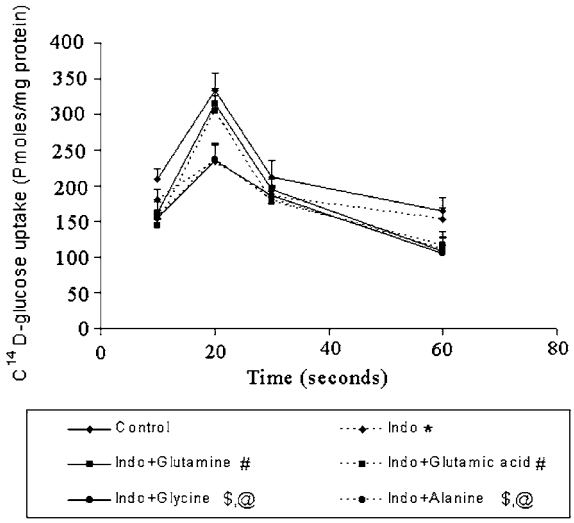

Figure 4 Effects on D-glucose uptake by BBMs from control rats and rats treated with indomethacin in the absence and presence of the different pretreatments at various time periods after incubation with radiolabelled glucose

Values represent means ± S.D. ($n = 6$ rats). Values at 20 s were compared among the groups. Overall P value for between-group comparison was $P < 0.001$. $^{*}P < 0.001$ compared with control; $\#P < 0.01$ compared with indomethacin (indo); $\$P < 0.01$ compared individually with indo + glutamine and indo + glutamic acid; $@P = 1$ compared with indo.

in the tricarboxylic acid cycle, driving mitochondrial ATP formation by oxidative phosphorylation. The ammonia produced readily diffuses into the portal blood and is extracted by the liver before it reaches the portal circulation, thus making the gut especially suited to metabolize glutamine.

Glutamine is known to contribute to the maintenance of the structural integrity of mucosal enterocytes. Studies have shown that utilization of glutamine by the gut increases after endotoxaemia and other stresses and appears to play a vital role in maintenance of the integrity of the gut during critical illness [38]. It has also been shown, by *in vitro* work using Caco-2 cells [39], that depletion of glutamine in the cells leads to increased bacterial translocation across epithelial cells. In fact, experimental data show that glutamine administration maintains gut barrier function in such situations [40]. In a number of injury models, improved survival has been demonstrated when enteral or intravenous feedings are supplemented with glutamine [9,10].

The enterocytes of the small intestine originate from the base of the crypt and migrate to the tip of the villi. They have the apparently paradoxical dual functions of facilitating absorption and at the same time maintaining an effective barrier to the entry of noxious substances from the intestinal lumen. At the apical surface of villus epithelial cells of the small intestine are found BBMs. These are in immediate contact with the contents of the lumen and form an important barrier between the lumen and the internal milieu of the epithelium. There is evidence to indicate that, under certain circumstances, the gut may lose its barrier function and allow entry of intestinal bacteria into the mucosa and into the systemic circulation. Such changes have been shown to occur in a variety of conditions, including burn trauma [41], surgical stress [42] and following ingestion of NSAIDs [43].

Ingestion of NSAIDs is known to result in increased intestinal permeability, resulting in the entry of bacteria and bile from the lumen into the mucosa. This aggravates the damage caused to the enterocyte by the topical effect of the drugs, leading to NSAID-induced enteropathy, a

condition in which there is chronic loss of blood and protein. One of the factors implicated in the pathogenesis of this condition, in addition to the inhibition of COX, is the decline in ATP levels in the cell, consequent upon inhibition or uncoupling of oxidative phosphorylation by NSAIDs [44]. The fall in ATP has been postulated to lead to relaxation of the tight junctions in the intestinal mucosa, thereby allowing noxious substances entry into the mucosa.

Another mechanism that may operate in the pathogenesis of NSAID-induced enteropathy is the alteration of the composition of the BBMs of the small intestine, a change that may lead to impaired barrier function. Such changes have been observed in intestinal BBMs from rats treated with indomethacin [8]. These effects have been postulated to contribute to the increased intestinal permeability that has been observed in response to the oral ingestion of NSAIDs and the consequent loss of barrier function [45]. In the present study, the administration of glutamine and glutamic acid was found to protect against oxidative stress in the BBMs of the intestinal mucosa. This protective effect may partly account for the observation that glutamine has been shown to attenuate the increase in intestinal permeability caused by NSAIDs [12,13].

Other mechanisms may also contribute to the beneficial effects of glutamine. The administration of glutamine and glutamic acid to the experimental animals was found to attenuate the oxidative stress in the intestinal mucosa, with resultant abolition of the effects of indomethacin on the mitochondria and the BBM vesicles. This effect was not seen as consistently with other isonitrogenous amino acids used, namely glycine and alanine. Thus glutamine and glutamic acid offer greater protection than glycine and alanine against indomethacin-induced mucosal damage.

The protection offered by glutamine and glutamic acid against such effects may be mediated by various mechanisms. One of these involves glutathione (GSH), which is an important cellular antioxidant. It is well known that glutathione is one of the major mechanisms involved in reducing oxidative stress in cells [46]. Glutamine and glutamic acid may serve as metabolic precursors for glutathione [47]. It has been shown in animal experiments that glutamine feeding preserved or even increased the GSH content in the intestine and liver after injury/ischaemia [48]. The importance of GSH is emphasized further by a study that showed a substantially disruptive effect on the mucosal architecture of pharmacological inhibition of GSH synthesis [49]. GSH is also known to be important for protection against chemical injury by serving as a substrate for glutathione transferase and glutathione peroxidase [50].

Another mechanism by which glutamine may exert its beneficial effects in the intestine may be by its conversion into citrulline [51], which then serves as a precursor for synthesis of arginine, the substrate for NO (nitric oxide) synthase, to form NO. NO is a potent vasodilator, an inhibitor of leucocyte activation and also a scavenger of free radicals produced by neutrophils [52]. The beneficial effects of NO are borne out by the fact that NO-NSAID derivatives have been shown to cause less damage to the gastrointestinal mucosa than the parent NSAIDs [53,54].

The protective effects of glutamine may also be related to the attenuation of proinflammatory cytokines subsequent to administration of the amino acid, thereby reducing cytokine release, organ damage and mortality [55]. Recently, it has been shown by *in vitro* studies that Caco-2 cells supplemented with glutamine can prevent cytokine-induced bacterial translocation. This was thought to be due to glutamine acting as an energy supplement, rather than through other mechanisms [39]. Such a hypothesis is borne out by studies that have shown that, in models of cell injury, supplementation with glutamine has been shown to raise levels of ATP in tissue [56–58]. This is also supported by studies that have documented the beneficial effects of glucose and citrate against NSAID-induced gastrointestinal damage, presumably by supplying substrates for the citric acid cycle [59,60].

Glutamine supplementation is also known to enhance induction of hsp70 (heat shock protein 70), expression of antioxidative HO-1 (haem oxygenase 1) and anti-apoptotic Bcl-2 molecules [61–63]. It has been reported [64] to enhance survival of activated T-cells by down-regulating CD95 and CD95L expression, and up-regulating CD45RO and Bcl-2 expression. Other studies of CD95-mediated caspase activities have shown that supplementation of glutamine significantly decreased caspase 3 and caspase 8 activities in activated T-cells [64]. Thus, in these studies, glutamine appears to protect the cells against apoptosis.

NSAIDs are commonly used in clinical practice on a long-term basis for the treatment of chronic inflammatory conditions. Long-term use, therefore, results in cumulative toxic effects of these drugs. The current model of small intestinal toxicity has been developed, using doses of 20 mg and 40 mg of indomethacin/kg of body weight, to attempt to mimic, in an acute model, the long-term effects of the drug. Both doses produced similar effects, with differences only in the degree of change (results not shown). The higher dose of indomethacin was used in the present study to assess the protective effect of glutamine. The fact that it is protective against even the higher dose shows that it is expected to be beneficial even when used at lower concentrations of the drug.

In the present study, the amino acids used were supplemented at a concentration of 2 %. This concentration was chosen as it was similar to that used to supplement total parenteral nutrition in human studies [65]. Other studies have also used this concentration of glutamine to

assess its potential as a protective agent in various animal models of tissue injury [66,67].

In conclusion, the present study has shown that oral supplementation with glutamine and glutamic acid prior to indomethacin administration prevents drug-induced alterations in the intestinal mucosa. Pretreatment with other isonitrogenous amino acids, such as glycine and alanine, was much less effective in this respect. Glutamine and glutamic acid appear to act by preventing indomethacin-induced oxidative changes in the intestinal mucosa and BBMs. It is possible that they do this by increasing the concentration of glutathione in the mucosal epithelial cells; however, other mechanisms may also exist. More work is required to elucidate the precise mechanism by which these amino acids confer protection against NSAID-induced small intestinal damage.

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